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# Use of Luminescent CdSe–ZnS Nanocrystal Bioconjugates in Quantum Dot-Based Nanosensors

P. T. TRAN (a), E. R. GOLDMAN (a), G. P. ANDERSON (a), J. M. MAURO (a), and H. MATTOUSSI[1]) (b)

*(a) Center for Bio/Molecular Science and Engineering, U.S. Naval Research Laboratory, Washington, DC 20375, USA*

*(b) Division of Optical Sciences, U.S. Naval Research Laboratory, Washington, DC 20375, USA*

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Biomolecules labeled with luminescent colloidal semiconductor quantum dots (QDs) have potential for use in numerous applications, including fluoro-immunoassays and biological imaging. QD labels exhibit size-tunable narrow-band luminescent emission and high resistance to photodegradation. They also exhibit efficient Förster energy transfer between neighboring QDs of different sizes and their emission is readily quenched by bound fluorescent dyes. In this paper, we describe preliminary results aimed at defining conditions for the design and preparation of nanoscale QD-bioconjugate sensors based on fluorescence quenching. We envision building sensor assemblies that employ quantum dots linked with dye-labeled biological receptors that utilize donor–acceptor energy transfer between QDs and receptors for conducting recognition-based assays. In particular, we report the effects of varying the concentration of energy acceptors bound to nanocrystal surfaces under both soluble and solid phase conditions on quenching phenomena.

**Introduction** Strong quantum confinement of the charge carriers in semiconductor nanocrystals (quantum dots, QDs) increases their effective band gap energy significantly with decreasing particle size, resulting in size dependence of QD light absorption and luminescence spectra [1–3]. Colloidal QDs are approximately spherical nanocrystals with surfaces that can be derivatized with a variety of functional capping groups (surface ligands), allowing their dispersion in a range of solvents, including aqueous environments. Several groups have shown that surface ligands can be used to attach various biological molecules to form QD-bioconjugates [4–7]. As an alternative to conventional fluorophores, QDs offer a number of attractive features, including high resistance to photodegradation, relatively narrow and symmetric luminescence bands, and simultaneous excitation of several sizes of QDs (thus several colors) over a broad range of wavelengths. Advantages in using luminescent QDs to synthesize bioconjugates with potential use in biological imaging applications and in developing QD-based nanosensors have been elaborated [4–6].

Our recent work has focused on the design and preparation of aqueous-compatible dithiol-capped CdSe–ZnS QDs having carboxylic acid terminal groups linked with bifunctional asymmetrically charged fusion proteins for QD-bioconjugate formation where conjugation is driven by electrostatic self-assembly [6]. In previous works, we studied the formation of QD-bioconjugates with two proteins, *E. coli* maltose binding

[1]) Corresponding author; Phone: 1-202-767-9473; Fax: 1-202-404-8114; e-mail: hedimat@ccs.nrl.navy.mil

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protein (MBP) and the IgG binding B2 domain of streptococcal protein G (pG), each genetically engineered to contain a basic (positively charged) leucine zipper interaction domain [6–8]. Using this approach, we successfully prepared QD/protein bioconjugates that retained both the photophysical properties of the QDs and the folded structure and molecular recognition functions of the attached proteins.

Fluorescence resonance energy transfer (FRET) involves nonradiative transfer of excited state energy from a donor to an acceptor molecule and can occur efficiently when appreciable overlap exists between the emission spectrum of the donor and the absorption spectrum of the acceptor [9, 10]. Earlier observations of efficient Förster energy transfer between neighboring closely packed colloidal QDs of different sizes (with different band gaps) [11] suggested that quenching of QD luminescence by surface-bound acceptor dyes in QD-bioconjugates would be feasible. In the present study, we investigated fluorescence quenching effects in model QD/protein assemblies where the attached proteins had been labeled with an organic quencher dye. Solution phase results showed that QD emission in these complexes could be progressively reduced and nearly eliminated at optimal QD/dye ratios. Under solid phase conditions effective quenching in an immobilized antibody system was also observed, providing insights for future efforts aimed at QD-based sensor development.

**Materials Preparation And Experimental Details** Stable water-compatible CdSe–ZnS QDs were prepared by derivatizing nanocrystal surfaces with dihydrolipoic acid (DHLA) as described in previous papets [6, 7]. Two-domain recombinant *E. coli* Maltose Binding Protein-basic zipper fusion protein (MBP-zb) and the dimeric bifunctional protein G-basic zipper (PG-zb) molecular adaptor were constructed and prepared essentially as described previously [6–8].

For fluorescence quenching assays using MBP protein, a non-emitting dye, thiol-selective QSY-7 maleimide ($C_{48}H_{48}ClN_5O_6S$, from molecular probes) was used to label the MPB variant MBP-tb A75C, which was genetically engineered to contain a single cysteine within the maltose binding protein sequence (ala-75 → cys-75) [12] (Fig. 1). Typically, MBP-tb A75C (1 mg/ml in 0.25 M sodium phosphate buffer at pH 7) was labeled by adding a $CH_3CN$ solution of QSY-7 maleimide to a stirring solution of the reduced protein. The concentration of acetonitrile in the reaction was less than 5% (v/v) in order to prevent MBP-tb A75C denaturation and aggregation, while the final dye concentration was approximately ten times the molar protein concentration. The QSY-7-labeled product was freed from excess unbound labels by gel filtration. These conditions produced labeled proteins at an approximate ratio of one dye per MPB based on spectoscopic measurements (data not shown). Conjugation of the chimeric proteins and DHLA-capped QDs driven by electrosatic self-assembly was subsequently carried out in 10 mM sodium borate at pH 9.

For experiments employing QD/antibody conjugates, goat IgG was labeled with the amine-reactive QSY-7 succinimidyl ester ($C_{43}H_{39}ClN_4O_7S$, molecular probes) following the manufacturer's protocol, resulting in approximately two covalently bound dyes per IgG (Fig. 1).

Solutions for fluorescence quenching experiments were prepared by adding mixtures of QSY-7-labeled MBP-tb A75C and unlabeled MBP-tb at various concentrations to colloidal QDs present at 300 nM in 100 μl of 10 mM sodium borate pH 9. In this way, the MPB-tb (labeled + unlabeled) : QD molar ratio was held constant at 5 : 1 while the

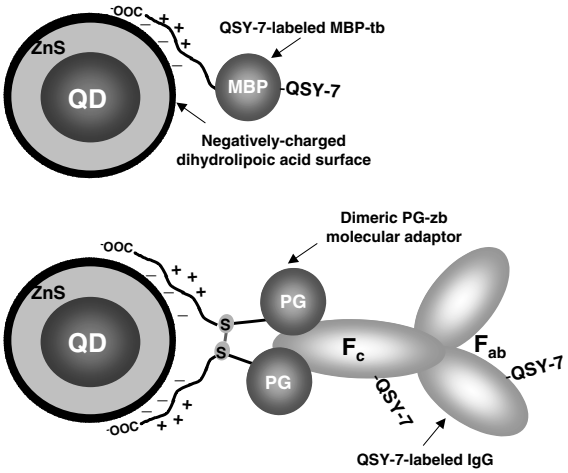


Fig. 1. Cartoons describing the conjugates used in quenching studies. The top drawing shows a QD-conjugated to QSY-7-labeled MBP-tb A75C. Bottom figure shows a QSY-7 labeled QD-IgG conjugate formed using the dimeric PG-zb. QSY-7 labeled sites are indicated for illustration only; the amine-reactive QSY-7 derivative used likely results in essentially random labeling mainly at surface-exposed lysine residues. For clarity, only a single protein or protein complex is shown on each QD; actual conjugates have multiple proteins bound to each nanocrystal [6, 7]

proportion of labeled MBP protein was varied. Thus, a ratio QSY-7–MBP-tb : QD = 0 (see Fig. 2) refers to a reaction in which only unlabeled MPB-tb was used, while at a QSY-7–MBP-tb : QD ratio of 5 only QSY-7-labeled MBP-tb A75C was used to form the conjugates. Reactions were incubated at room temperature for 15 min, diluted with 10 mM sodium borate buffer to 600 μl, and emission spectra of the bioconjugate solutions were collected on a SLM 6100 fluorometer (Aminco) with excitation at 340 nm.

Surface-immobilized DHLA QDs were prepared using poly-L-lysine coated microscope slides (Sigma). QDs were applied on slide surfaces in drops containing approximately 1 pmol of nanocrystals in 5 μl of 1X TBE buffer (89 mM tris borate, 20 mM EDTA), then incubated for 1 h in a humid chamber to prevent the drops from drying. Unbound QDs were then removed from spots by first drawing off excess liquid with a micropipet, and then by rapidly immersing slides in excess TBE buffer. After shaking off excess buffer and a brief water rinse, the slides were allowed to dry briefly at room temperature. For quenching studies using QSY-7 labeled IgG antibodies, spots of immobilized nanocrystals were first treated for 1 h with 5 μl volume of saturating solutions of the dimeric PG-zb molecular adaptor we have previously described [8], washed and dried as above, then QSY-7 labeled IgG was added in 5 μl drops to the same spots, and

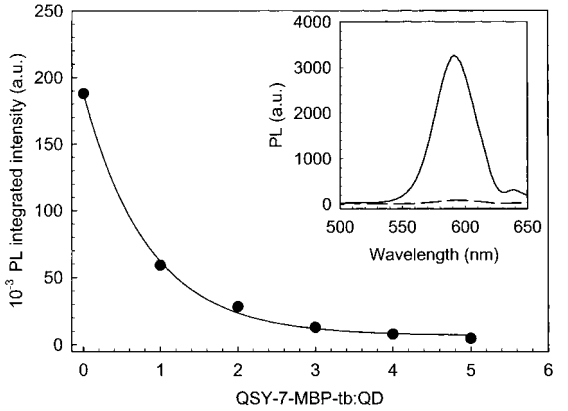


Fig. 2. PL integrated intensity vs. increasing molar ratio of QSY-7–MBP-tb to QD. In this experiment, the total numbers MPB-tb protein (labeled + unlabeled) and QDs remained fixed (protein to QD ratio of 5), while the labeled : unlabeled MBP-tb ratio varied (see text). The solid line is an exponential decay fit to the data. Inset shows PL spectra of solutions in a 2 mm optical path cell containing 30 pmol QDs and 100% unlabeled MPB-tb (solid line) (QSY-7–MBP-tb: QD = 0), and 30 pmol QDs and 100% QSY-7 labeled protein (dashed line) (QSY-7–MBP-tb : QD = 5)

each was allowed to incubate for an additional hour, followed by removal of excess protein, slide washing and air-drying. Fluorescence emission was monitored using UV epi-illumination excitation at 254 nm and a 523 nm long pass emission filter, utilizing a Kodak Image Station 440. The nanocrystals used in the present study had an emission maximum at 590 nm with a full width at half maximum (FWHM) of ~35 nm.

**Results and Discussion** QSY-7 was chosen as a non-emitting quenching chromophore for the present experiments due to its broad absorption spectrum that fully overlaps with the QD emission. Its relatively broad absorption spectra should permit its use with available blue-, green-, orange- or red-fluorescent CdSe–ZnS quantum dot donors. Figure 2 shows the decay of QD photoluminescence (PL) from QD-bioconjugate solutions as a function of increasing the average number of QSY-7 labeled proteins attached to each nanocrystal. Quenching of the nanocrystal emission occurs systematically with increasing amounts of quencher-labeled protein bound, with the nanocrystals losing about 90% of their signal when ~3 out of 5 MBP-tb molecules were labeled with QSY-7. As shown, when all the MBP molecules used to form conjugates were labeled with the QSY-7 quencher, nearly all QD emission was quenched (Fig. 2 and inset). To examine the extent of possible intermolecular quenching effects in these experiments, the highly quenched solutions containing QD-conjugates made with 100% QSY-7 labeled MBP-tb proteins were diluted over a tenfold range in order to reduce the "apparent concentration" of any residual unbound QSY-7 labeled proteins present in solution. No recovery in the PL signal upon dilution was observed, except for expected dilution effects that varied strictly linearly with the degree of dilution. These results strongly suggest that the observed quenching effects are due to radiationless energy transfer that occurs between QDs and stably bound MBP/QSY-7 (i.e., FRET).

A second system was investigated in which emission of surface-tethered QDs was monitored as QSY-7-labeled antibodies were bound to immobilized dots using a protein G molecular adaptor previously described [6, 7]. In this layer-by-layer approach to surface bound prototype sensor development, QDs were first immobilized on commercially obtained glass slides treated with poly-L-lysine. A second layer was generated by QD/PG-zb conjugate formation driven by electrostatic self-assembly, resulting in surface-bound quantum dots with high selectivity for the Fc region of IgG antibodies. Finally, QSY-7-labeled IgG antibodies were introduced onto these surfaces for binding to dots through the PG-zb molecular adaptor. Figure 3 shows luminescence from several stages of preparation of the prototype surface sensor on a single slide. Spot 1 shows the luminescence intensity of unconjugated immobilized DHLA-capped QDs. Spot 2 shows luminescence intensity of the QDs after their surfaces have been saturated with PG-zb. The increased intensity observed for spot 2 relative to spot 1 is attributed to the well-documented enhancement in the PL that invariably occurs when QDs are conjugated to proteins [6, 7], although the present observation is the first example reported of this effect occurring on a solid support. Spots 3 through 6 qualitatively demonstrate that fluorescence quenching occurs in a systematically increasing fashion as the proportion of QSY-7 labeled IgG molecules conjugated to surface-bound QD spots increases. The diminished but still readily observable QD emission that persisted at the higher relative proportions of QSY-7-labeled IgG molecules (spots 5 and 6 in Fig. 3) suggests that the full quenching effect possible under these conditions has been achieved (i.e. all available QD surfaces are saturated with labeled antibody). Obtaining

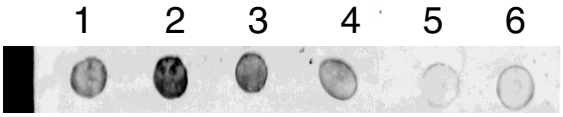


Fig. 3. Quenching of the QD PL in a layer-by-layer solid phase sensor construction prototype experiment. The spots 1–6 on the image show emission of QDs immobilized on the slide as follows: 1: unconjugated QDs, 2: QDs conjugated with PG-zb, 3: QD/IgG conjugates with 8X IgG (100% unlabeled IgG), 4: QD/IgG conjugates with 4X QSY-7 IgG, 5: QD/IgG conjugates with 6X QSY-7-IgG, and 6: QD/IgG conjugates with 8X QSY-7-IgG (100% QSY-7 labeled IgG). The intensity of spot 2 exceeds that of spot 1 due to enhancement of the QD luminescence that occurs upon conjugation with the proteins, as previously observed in solutions [6]

optimized levels of quenching in this type of system could involve a number of modifications, including more extensive labeling of antibodies with quencher, utilizing recombinant versions of antibodies that are smaller than intact IgGs to decrease dot-quencher distances, and altering QD immobilization conditions to maximize nanocrystal surface availability for protein binding. Nonetheless, these observations of quenching using the layered solid phase approach are qualitatively analogous to the homogeneous soluble cases previously explored (above). Developing the means to prepare QD-based sensors on solid surfaces, based on results of prototype studies such as this one, will greatly enhance the usefulness of this type of detection strategy, especially in diagnostic assay and sensor device applications.

Further characterization of the quenching properties of these and other QD-bioconjugate complexes is underway, including exploration of effects of changing the nanocrystal size to tune the emission, the type of quenching dye used, the degree of overlap between the light absorption bands of acceptors and the PL emission bands of the QD donors, distance and orientation factors, as well as examination of details of the quenching mechanism(s) taking place in these systems.

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